

# A retroviral packaging cell line for pseudotype vectors based on glioma-infiltrating progenitor cells

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Received: 6 October 2006  
Revised: 5 February 2007  
Accepted: 19 February 2007

## Abstract

**Background** Early clinical trials for gene therapy of human gliomas with retroviral packaging cells (PC) have been hampered by low transduction efficacy and lack of dissemination of PC within the tumor. In the current approach, these issues have been addressed by creating a stable packaging cell line for retroviral vectors pseudotyped with glycoproteins of lymphocytic choriomeningitis virus (LCMV) based on tumor-infiltrating progenitor cells.

**Methods** Tumor-infiltrating progenitor cells, which had been isolated from adult rat bone marrow (BM-TIC), were modified to stably express Gag-Pol proteins of moloney murine leukemia virus (Mo-MLV) and glycoproteins of LCMV. Packaging of a retroviral vector was measured by titration experiments on human fibroblast cells as well as on mouse and human glioma cell lines. Additionally, gene transfer was tested in a rat glioma model *in vivo*.

**Results** The BM-TIC-derived packaging cell line (BM-TIPC) produced retroviral vectors with titers between  $2-8 \times 10^3$  transducing units (TU)/ml. Extended culturing of BM-TIPC over several weeks and freezing/thawing of cells did not affect vector titers. No replication-competent retrovirus was released from BM-TIPC. In a rat glioma model, BM-TIPC infiltrated the tumors extensively and with high specificity. Moreover, BM-TIPC mediated transduction of glioma cells *in vivo*.

**Conclusion** This proof-of-principle study shows that primary adult progenitor cells with tumor-infiltrating capacity can be genetically modified to stably produce retroviral LCMV pseudotype vectors. These BM-TIPC may be a useful tool to enhance specificity and efficacy of gene transfer to gliomas in patients. Copyright © 2007 John Wiley & Sons, Ltd.

## Introduction

Malignant gliomas are the most frequent primary brain tumors in adults and are associated with a high mortality rate [1]. These tumors are typically highly resistant to radiation and chemotherapy. Moreover, the infiltrative growth pattern of gliomas does not allow complete tumor resection [2].

Early gene therapy trials were initiated to find alternative treatment strategies to relieve the dismal prognosis of glioma patients. In these trials, retroviral vectors were produced *in situ* by packaging cells (PC) grafted into the tumor or the resection cavity. The retroviral vectors produced by these PC encoded the suicide gene herpes simplex virus thymidine kinase (HSV-tk), which renders transduced tumor cells sensitive to

the prodrug ganciclovir. Despite very promising results in animal models [3–5], these trials failed in the clinical setting [6,7]. The major reason for this failure was that the mode of vector delivery did not allow efficient transduction of larger tumors or specific targeting of infiltrating glioma cells [8].

Neural stem cells (NSC) have been proposed for delivery of therapeutic molecules *in vivo*. NSC infiltrate gliomas *in vivo* after intratumoral injection and colocalize with invasive tumor cells [9–11]. Similar to NSC, stem cells of non-neural origin have shown to specifically infiltrate gliomas without migrating into normal brain tissue [12–14]. Accordingly, we recently found that a subpopulation of mesenchymal stem cells (MSC) infiltrate gliomas *in vivo* with high efficacy and specificity (Miletic, Fischer *et al.*, submitted). This cell type was isolated from adult rat bone marrow according to the protocol for generating multipotent adult progenitor cells (MAPC) published by Jiang and colleagues [15]. We subsequently referred to these cells as bone-marrow-derived tumor-infiltrating cells (BM-TIC). Unlike most types of adult progenitor cells, BM-TIC have a very high expansion capacity *in vitro*. Rapid and extensive expansion is required to create retroviral packaging cell lines.

Provided that both efficient and glioma-specific delivery of therapeutic vectors can be achieved, another critical step is the efficient entry of the vector into the glioma cells. In previous studies on glioma gene therapy, retroviral vectors carried the amphotropic murine leukemia virus (MLV) envelope protein. However, this envelope allows transduction of human glioma cells with only moderate efficiency. In contrast, transduction of glioma cells can be enhanced by up to tenfold, if the amphotropic MLV envelope protein is exchanged by the glycoproteins of LCMV (LCMV-GP). Importantly, LCMV-GP is not cytotoxic, so that stable and constitutive expression of LCMV-GP in PC can be achieved [16,17]. Above all, LCMV-GP-mediated gene transfer to normal brain cells is extremely inefficient so that LCMV-GP pseudotypes transduce glioma cells in the brain with great specificity [17].

The present work describes the generation of stable PC for retroviral LCMV-GP-pseudotyped vectors based on BM-TIC. To establish this cell line, BM-TIC were genetically modified to express Mo-MLV Gag-Pol proteins and LCMV-GP. Viral vectors were produced in bulk cultures of BM-TIC packaging cells (BM-TIPC) with titers of  $1-8 \times 10^3$  transducing units (TU)/ml. BM-TIPC were passaged *in vitro* over several weeks without loss of vector titer. Replication-competent retrovirus (RCR) was not detected in BM-TIPC. Moreover, BM-TIPC infiltrated gliomas *in vivo* and mediated transduction of glioma cells. This report is the first to show that PC for LCMV-GP-pseudotyped retroviral vectors can be derived from tumor-infiltrating progenitor cells and that these BM-TIPC retain their specific migratory capacity and can be used for specific vector delivery to gliomas *in vivo*.

## Materials and methods

### Cell lines

The human embryonic kidney cell line 293T (ATCC number CRL-11268), the human rhabdomyosarcoma cell line TE671 (ATCC number CCL-136) and the rat gliosarcoma cell line 9L (ATCC number CRL-2200) were obtained from the American Type Culture Collection (ATCC). 9L-DsRed cells were established by transduction of 9L cells with the retroviral DsRed-encoding vector pMP71DsRed as described previously [17]. The human glioblastoma cell line G62 was a gift from Prof. M. Westphal, University Hospital Hamburg, Germany. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% glutamine and grown at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Rat MSC were kindly provided by Dr. C. Lange, UKE Hamburg, Germany, and were cultured in Ham's-F12 medium (Promocell, Heidelberg, Germany) supplemented with 20% FCS.

### BM-TIC isolation and characterisation

BM-TIC were prepared from bone marrow of rats following a protocol previously described [15], with minor modifications. Fisher 344 rats (4–6 weeks old, female) were killed and femurs and tibias were collected. Bone marrow tissue was flushed out using an 18G syringe. A single-cell suspension was obtained and seeded into a T-75 flask covered with fibronectin (Sigma). Non-adherent cells were removed after 3 days of culture. Culture medium consisted of 60% DMEM/LG (Gibco), 40% MCDB-201 (Sigma) with 2% fetal bovine serum (FBS) (Pan Biotech), penicillin/streptomycin, 50 nM dexamethasone (Sigma),  $10^{-4}$  M ascorbic acid 2-phosphate (Sigma), linoleic acid-bovine serum albumin 0.5 mg/ml (Sigma),  $1 \times$  insulin transferrin selenium (Gibco), and was supplemented with 10 ng/ml platelet-derived growth factor-BB (Sigma), 10 ng/ml epidermal growth factor (Sigma) and 1000 U/ml leukemia inhibitory factor (Chemicon). Cells were grown at 37 °C in 5% CO<sub>2</sub> and maintained at low density by subculturing the cells every third day.

### Antibody staining and flow cytometric analysis of BM-TIC and MSC

Rat-specific antibodies against CD44 (clone Ox-49), CD45 (clone Ox-1) and MHC class I (clone Ox-18) were purchased from BD Pharmingen (Heidelberg, Germany). For flow cytometric analysis,  $3 \times 10^5$  cells were incubated for 30 min with 1  $\mu$ g of primary antibody at room temperature. Cells were then washed three times with phosphate-buffered saline (PBS) containing 3% FBS and incubated for an additional 20 min with the appropriate APC-conjugated secondary antibody in a dilution of 1 : 50.

Cell-surface expression of the respective antigens was analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

### Construction of the lentiviral SIN vector encoding LCMV-GP

A synthetic LCMV-GP gene with optimized codons was obtained from Geneart GmbH, Regensburg, Germany. The LCMV-GPopt gene was cloned into EcoRI and NotI sites of pLCMV-GP-IRES-Puro, which was a gift from Dr. Winfried Beyer, University of Kiel, Germany [18]. The LCMV-GPopt/IRES/Puro cassette was inserted into pBluescript (Stratagene). From there, the LCMV-GPopt/IRES/Puro cassette was excised by BamHI and XhoI digestion and ligated into pHR'SIN-SEW to replace the eGFP gene [19]. The resulting lentiviral self-inactivating (SIN) vector pHR'SIN-GPopt contained an internal spleen focus forming virus (SFFV) promoter and allowed coexpression of LCMV-GPopt and the puromycin resistance gene (PuroR) via the internal ribosomal entry site (IRES).

### Retroviral vector production by transient transfection of 293T cells

The 293T cell line was used for transient production of gammaretroviral or lentiviral vectors. For production of gammaretroviral vectors, the retroviral vector plasmid MP71-eGFP-wPRE [20] was cotransfected with the Gag-Pol expression plasmid pSV-Mo-MLVgagpol and the envelope expression plasmid pHCMV-LCMV-GP [16] into 293T cells. Lentiviral vectors were generated by cotransfecting 293T cells with the lentiviral vector plasmid pHR'SIN-GPopt (5  $\mu$ g), the HIV Gag-Pol expression plasmid pCMV-dR8.91 (15  $\mu$ g) [21] and pHCMV-LCMV-GP (2  $\mu$ g). Forty-eight hours after transfection, cell culture supernatants containing retroviral vectors were collected and filtered through 45  $\mu$ m pore size filters. Vector supernatants were used directly to infect BM-TIC and other cell lines, or stored at  $-80^{\circ}\text{C}$ .

### Titration of retroviral vectors

Titration of gammaretroviral and lentiviral LCMV-GP pseudotype vectors was performed on TE671 cells. Titration of vectors produced in BM-TIPC was additionally performed on 9L and G62 cells. Serial dilutions of vector supernatants were prepared and 0.5 ml of each dilution was added to  $5 \times 10^4$  cells seeded in a well of a 24-well plate. Plates were centrifuged for 1 h at 1000 g. For titration of vectors carrying the enhanced green fluorescent protein (eGFP) gene, cells were analyzed for eGFP expression 65 h after transduction by flow cytometry. For vectors carrying the LCMV-GP gene, cells were stained with a LCMV-GP-specific antibody as described below and analyzed by flow cytometry. All flow cytometry measurements were performed on

a FACSCalibur (Becton Dickinson) using the CellQuest Pro software. Titers were calculated from dilutions which resulted in 0.5 to 20% of transduced cells. Within this range, a linear correlation between vector input and percentage of transduced cells can be expected.

### Generation of a BM-TIC packaging cell line

For stable transfection of BM-TIC with the Mo-MLV Gag-Pol expression plasmid pGag-Pol-IRES-bsr [18], cells were seeded into 24-well plates at  $1 \times 10^3$  cells/well 2 days before transfection. Transfection was performed using the lipophilic reagent TransFectin (BioRad, Germany) according to the manufacturer's instructions. For each well, 1  $\mu$ g of plasmid DNA was used for transfection. Cells were incubated with DNA for 6 h, after which medium was changed. Forty-eight hours after transfection, BM-TIC were trypsinized, plated into 10 cm dishes and maintained in selection medium containing blasticidin (5  $\mu$ g/ml). After 10 days, colonies were harvested using sterile Q-tips and maintained as individual clones in selection medium.

For transduction of BM-TIC with the lentiviral vector pHR'SIN-GPopt or the gammaretroviral vector MP71-eGFP-wPRE, BM-TIC were plated into 24-well plates at  $5 \times 10^3$  cells/well on the day before transduction. The following day, medium was replaced with vector-containing supernatants. Plates were centrifuged for 1 h at  $37^{\circ}\text{C}$  and 1000 g. Fresh medium was added 6 h after transduction. For BM-TIC transduced with pHR'SIN-GPopt, cells were trypsinized 48 h after transduction and plated into 10 cm dishes in selection medium containing puromycin (2.5–5  $\mu$ g/ml). For BM-TIC transduced with MP71-eGFP-wPRE, cells were analysed for eGFP expression 48 h after transduction by flow cytometry.

BM-TIC packaging cells (BM-TIPC) expressing both pGag-Pol-IRES-bsr and pHR'SIN-GPopt were permanently maintained under selection with puromycin and blasticidin (5  $\mu$ g/ml each). To test vector production in BM-TIPC, cells were seeded into 6-well plates at  $5 \times 10^4$  cells/well in 2 ml of medium without selection antibiotics. The next day, cell culture medium of BM-TIPC was used to transduce TE671 cells as described above.

### Flow cytometric analysis of Gag and LCMV-GP expression

Expression of Mo-MLV Gag and LCMV-GP in BM-TIC was assessed by flow cytometric analysis. For Gag detection,  $1 \times 10^6$  cells were harvested, fixed and permeabilized using the Fix&Perm Kit (Caltag). Staining for intracellular Gag was performed with a polyclonal anti-p30 rabbit serum, which was kindly provided by Dr. Carol Stocking, University Clinic Hamburg-Eppendorf, Germany. The secondary antibody was a phycoerythrin (PE)-conjugated Fab-anti-rabbit (Caltag). For LCMV-GP detection,  $5 \times 10^5$  BM-TIC were harvested and fixed with 3% formaldehyde.

LCMV-GP on the cell surface was stained with the monoclonal antibody KL25 [22] and the PE-conjugated goat-anti-mouse serum (Caltag).

### Test for replication-competent retrovirus

A sensitive polymerase chain reaction (PCR)-based assay was applied to test the presence of replication-competent retrovirus (RCR) in BM-TIPC supernatants. TE671 cells were plated into 24-well plates at  $5 \times 10^4$  cells/well and transduced with 0.5 ml supernatant of BM-TIPC. As positive control, TE671 cells were transduced with supernatants from cells infected with amphotropic MLV (A-MLV). TE671 cells were then incubated for 9 days. After this period, cell culture supernatants from TE671 cells were used to infect fresh TE671 cells. After 48 h, TE671 cells were harvested, genomic DNA was isolated using the DNeasy Kit (Qiagen) and used as templates for PCR. Primers pairs used to amplify actin, Mo-MLV Gag, amphotropic MLV envelope protein (A-MLVenv) and LCMV-GP sequences were: actin 5' GCT CGT CGT CGA CAA CGG CTC 3' and 5' CAA ACA TGAT CTG GGT CAT CTT CTC 3'; Mo-MLV Gag 5' CTT CCT AGA GAG ACT TAA GG 3' and 5' GTT GGG ACC TCC TTC GTT CTC 3' [23]; A-MLVenv 5' TGT AAC CTG GAG AGT CAC CAA C 3' and 5' CTC CGA CCA GAT CAC ATA GAT C 3'; LCMV-GP 5' GCA GCC AGA CCA GCT ACC 3' and 5' GCT GTT CAC GGT GGT CTT G 3'. The annealing temperature was 53 °C. For each reaction, 40 amplification cycles were performed. PCR products were subjected to agarose gel electrophoresis in the presence of ethidium bromide and visualized under UV light.

### Animal experiments

Adult female Fisher 344 rats (Harlan Winkelmann, Borchon, Germany) were anesthetized with intraperitoneal (i.p.) injection of ketamine (50 mg/kg) and xylazine (2 mg/kg). Intracranial 9L-DsRed tumors were established by injection of  $8 \times 10^4$  9LDsRed cells (in 2 l PBS) into the right striatum using a Hamilton syringe in a stereotaxic apparatus (Stoelting, IL, USA). The coordinates used were 4 mm lateral to the bregma and 5 mm in depth to the dural surface. Five days after tumor implantation, rats were anesthetized, and  $4 \times 10^5$  BM-TIPC were injected using the same stereotactic coordinates. For control experiments, BM-TIC expressing only the gammaretroviral eGFP vector or the eGFP vector and the lentiviral LCMV-GP vector were injected into rat tumors as described above. After 7 days, animals were euthanized and perfused with 4% paraformaldehyde. Brains were removed, suspended in 30% sucrose for 3 days, and then snap-frozen in isopentane chilled with liquid nitrogen. Coronal sections (12  $\mu$ m) were prepared on a cryostat and examined under a fluorescence microscope (Zeiss, Jena, Germany). In addition, sections were analyzed by confocal scanning laser microscopy (Leica, UK)

to detect *in vivo* transduction of tumor cells. Transduction efficiencies in infected tumor areas were determined by counting cells on confocal microscopic images from three different animals.

## Results

### Isolation and characterization of BM-TIC

Tumor-infiltrating progenitor cells were isolated from adult rat bone marrow following a protocol established by Jiang *et al.* [15] to derive multipotent adult progenitor cells (MAPC). BM-TIC proliferated with a population doubling time of approximately 24 h and could be expanded for more than 60 passages without apparent signs of senescence (data not shown). To further characterize BM-TIC, expression of major histocompatibility complex class I (MHC I), CD44 and CD45 was assessed. As shown in Figure 1, BM-TIC are negative for MHC I, CD44 and CD45, thereby resembling MAPC [15]. In contrast, rat MSC express both MHC I and CD44, as previously reported [24].

### Genetic modifications of BM-TIC to create a packaging cell line for gammaretroviral LCMV-GP pseudotypes

To create a BM-TIC-based packaging cell line (BM-TIPC) for LCMV-GP pseudotypes, Mo-MLV Gag-Pol and LCMV-GP genes were introduced into BM-TIC. As a first step, BM-TIC were transfected with the Mo-MLV *gag-pol* expression plasmid pGag-Pol-IRES-bsr (Figure 2), which coexpresses a blasticidin resistance gene and Mo-MLV *gag-pol* via an IRES. Clones were grown in selection medium and examined by flow cytometry for Mo-MLV Gag expression. Gag expression in clone BM-TIC-C1 is shown in Figure 3A. To achieve stable LCMV-GP expression in BM-TIC, the lentiviral self-inactivating (SIN) vector pHR'SIN-GPopt with internal SFFV promoter and LCMV-GP/IRES/Puro cassette was created (Figure 2). BM-TIC-C1 were transduced with pHR'SIN-GPopt and subsequently transferred to selection medium. Transduced BM-TIC were expanded as bulk cultures without isolating single clones. LCMV-GP expression in BM-TIC-C1/GP was confirmed by flow cytometry at two different time points after transduction. As shown in Figure 3B, LCMV-GPopt expression was detectable in BM-TIC-C1/GP and remained stable during 10 weeks of culture. The gammaretroviral vector MP71-eGFP-wPRE (Figure 2) was then introduced by transduction into BM-TIC-C1/GP to create BM-TIPC. Expression of the eGFP was determined by flow cytometry and was present in >90% of cells (data not shown).

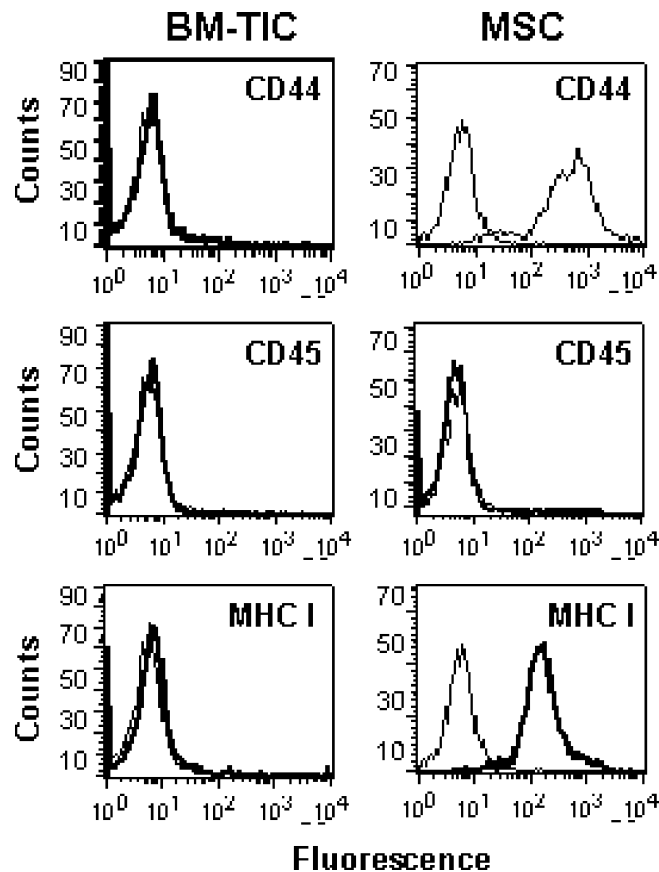


Figure 1. Staining of BM-TIC and MSC for cell-surface antigens. BM-TIC and MSC were stained with antibodies against CD44, CD45 or MHC I and with APC-conjugated secondary antibodies. Control samples were stained with secondary antibody only. Samples were analyzed by flow cytometry

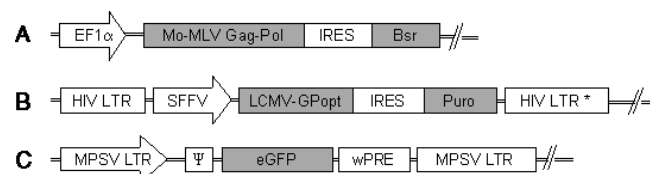


Figure 2. Packaging constructs used to establish BM-TIPC. Promoters are shown as white arrows, open reading frames as grey boxes. The bicistronic expression plasmid pGag-Pol-IRESbsr (A) encodes Mo-MLV Gag-Pol and confers blasticidin resistance (bsr) to stable transfectants. The bicistronic lentiviral vector pHR'SIN-Gpopt (B) encodes a codon optimized LCMV-GP together with a puromycin resistance gene (Puro). The 3' HIV LTR has a deletion in the U3 region, which leads to self-inactivation of the promoter activity of the HIV LTR during reverse transcription. This prevents remobilization of the integrated lentiviral vector from the host cell. Transgene expression from pHR'SIN-Gpopt is driven by an internal SFFV promoter. The gammaretroviral reporter construct MP71-eGFP-wPRE (C) contains intact LTRs and the gammaretroviral packaging signal  $\Psi$  and encodes the enhanced green fluorescent protein (eGFP). EF1a: elongation factor 1a promoter; IRES: internal ribosomal entry site from Encephalomyocarditis virus; LTR: long terminal repeats; MPSV: myeloproliferative sarcoma virus; SFFV: U3 promoter/enhancer region of the spleen focus forming virus; wPRE: woodchuck hepatitis virus posttranscriptional regulatory element

### Vector production in BM-TIPC and transduction of glioma cell lines *in vitro*

Efficiency and stability of vector production in BM-TIPC was tested by titration of supernatants on TE671 cells. Vector titers ranged between  $1.3 \times 10^3$  and  $7.9 \times 10^3$  TU/ml and remained stable over 1 month in culture and after several cycles of freezing/thawing of BM-TIPC (Figure 4A). Vector titers on 9L rat glioma cells and G62 human glioma cells were subsequently measured. Vectors

produced in BM-TIPC mediated transduction of both glioma cell lines *in vitro*. The efficiency of transduction of 9L cells was comparable to TE671, whereas G62 human glioma cells were transduced with about 4-fold higher efficiency (Figure 4B).

The presence of replication-competent retrovirus (RCR) in BM-TIPC supernatants was tested in a PCR-based assay (refer to 'Material and methods' section), which in previous studies reproducibly detected RCR in positive controls (i.e. cell lines producing amphotropic replication-competent MLV) and which is more sensitive than

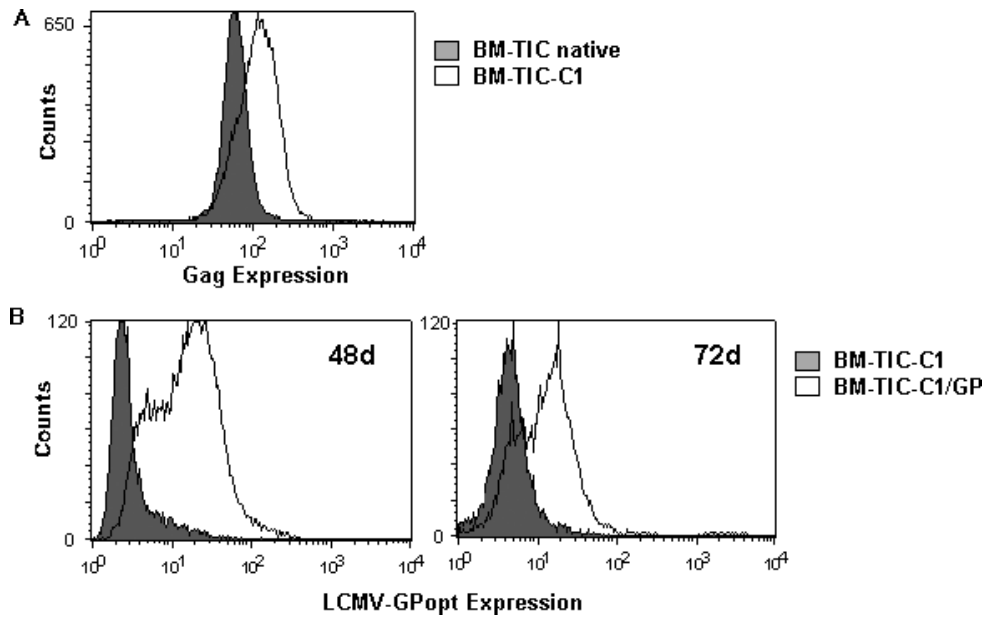


Figure 3. Expression of MLV Gag and LCMV-GPopt in BM-TIC. (A) Gag expression in BM-TIC after stable transfection with pGag-Pol-IRES-bsr was determined by intracellular staining with an anti-p30 antibody and a PE-conjugated secondary antibody. (B) Expression of LCMVGP in BM-TIC-C1 after transduction with pHR'SIN-GPopt was determined by staining of the cell surface with an a-LCMV-GP primary antibody (KL25) and a PE-conjugated secondary antibody. Staining against LCMV-GP was performed at two different time points (day 48 and day 72) after transduction. Samples were analyzed by flow cytometry

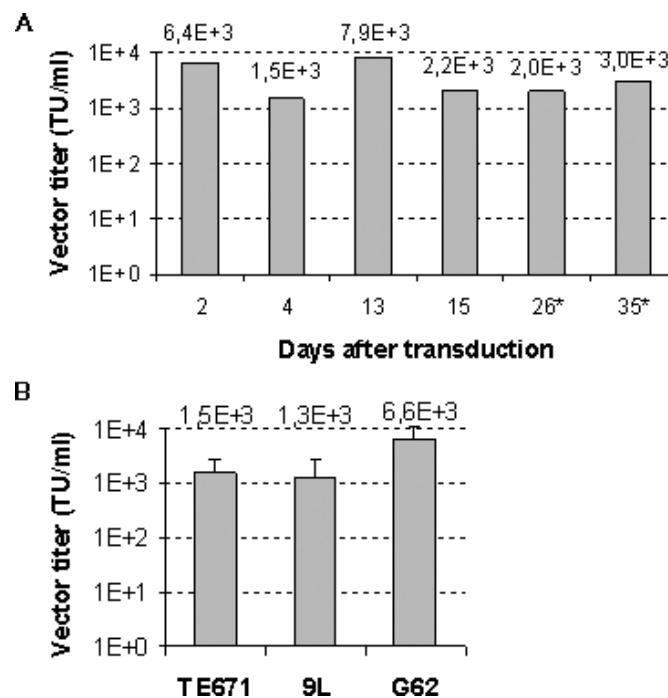
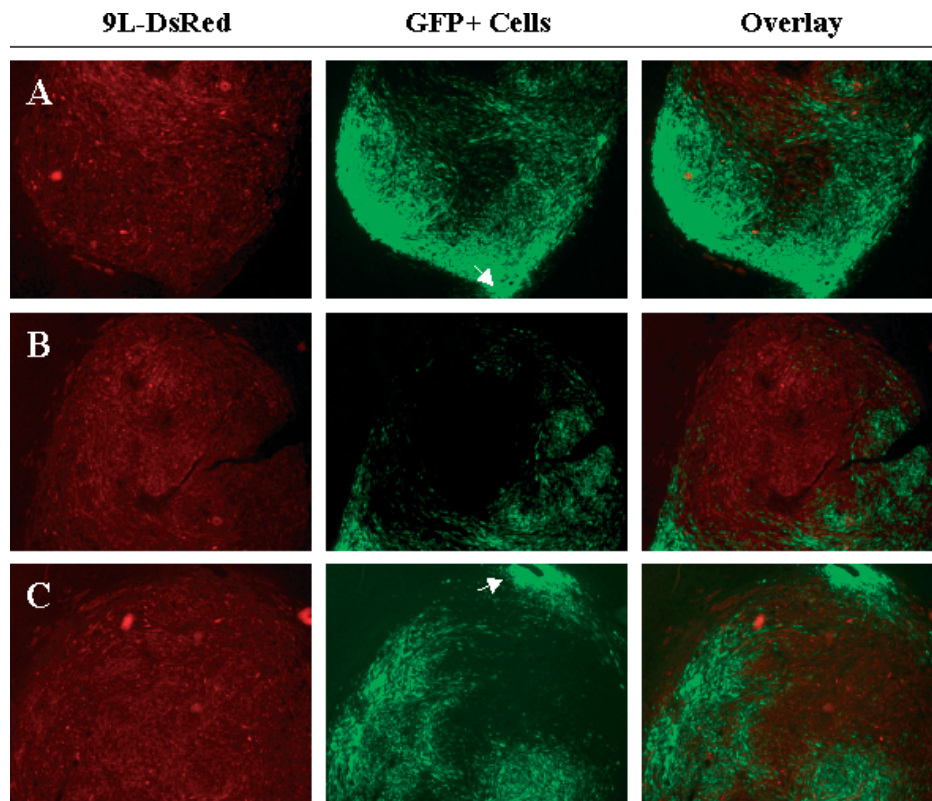


Figure 4. Production of retroviral vectors by BM-TIPC. (A) Cell culture supernatants from BM-TIPC were used to transduce TE671 cells at indicated time points after transduction with MP71-eGFP-wPRE. (\*) BM-TIPC had undergone three cycles of freezing/thawing. (B) Vectors produced in BM-TIPC were titrated on TE671, 9L rat glioma and G62 human glioma cell lines in parallel. Data are shown as mean values and standard deviations from three independent experiments

the commonly used marker rescue assay (our own unpublished observations). BM-TIPC supernatants were free of any detectable RCR, as demonstrated by lack of MLV Gag, A-MLVenv and LCMV-GPopt sequences in genomic DNA of TE671 indicator cells (Supplementary Figure S1, see Supplementary Material).

### Glioma-specific migration of BM-TIPC and transduction of glioma cells *in vivo*

For generation of the BM-TIPC, foreign (viral and non-viral) genes were introduced and the cells were expanded for numerous passages *in vitro*. The ability of these



**Figure 5.** BM-TIPC infiltrate gliomas *in vivo* with high specificity and efficacy. BM-TIPC were injected intratumorally into established 9L-DsRed gliomas in Fischer rats. After 7 days, rats were killed, brain sections were prepared and analyzed by fluorescence microscopy. Panels A, B and C represent sections from three different tumors injected with BM-TIPC. Magnification 2.5 $\times$ , white arrows indicate the site of injection of BM-TIPC

extensively manipulated BM-TIPC to specifically infiltrate gliomas *in vivo* was analyzed. BM-TIPC were injected into established 9L-DsRed gliomas in Fischer rats. After 7 days, examination of brain sections revealed an efficient infiltration of gliomas by BM-TIPC (Figure 5). BM-TIPC migrated far away from the injection site (indicated by arrows in Figures 5A and 5C) and infiltrated large proportions of the tumor. Moreover, the migratory behavior of BM-TIPC was highly specific for gliomas, as no BM-TIPC infiltration into normal brain was observed. Transduction of glioma cells *in vivo* was examined by confocal microscopy on the same brain sections. Colocalization of green and red fluorescence, indicative of transduced glioma cells, was detected in both solid (Figure 6A) and infiltrating (Figure 6B) tumor areas. In contrast, when BM-TIC expressing the gammaretroviral eGFP vector alone or together with LCMV-GP but lacking Mo-MLV Gag-Pol were injected into rat gliomas, no presence of red and green fluorescence in the same cell could be detected (Figures 6D and 6E). This shows that only BM-TIPC with complete packaging function can transfer the retroviral vector to glioma cells and that the colocalization of red and green fluorescence was not a result of cell fusion or uptake of fluorescent proteins from surrounding cells. The percentage of transduced glioma cells reached up to 33% in some tumor areas. This shows that BM-TIPC continue to produce retroviral vectors *in vivo* and facilitate glioma cell transduction.

Importantly, BM-TIPC were also found adjacent to single infiltrating glioma cells and mediated their transduction (Figure 6C, arrow).

## Discussion

The success of gene therapy for gliomas depends primarily on the efficient and glioma-specific transfer of the therapeutic gene. By injecting fibroblast-based packaging cells (PC), efficient tumor cell transduction can be achieved in animal models but not in clinical settings, due to the non-migratory character of these PC [6]. Stem cells of neural and non-neural origin can be used to deliver therapeutic molecules to gliomas *in vivo* [9,10,12]. However, the expression of toxic genes only in the tumor-infiltrating cells but not the tumor itself may not be sufficient to achieve a sufficient therapeutic effect for the treatment of human gliomas. The aim of the present work was therefore to create a PC based on glioma-infiltrating progenitor cells. Most adult progenitor cells, like neural and mesenchymal stem cells, however, have only limited expansion capacity unless immortalized with oncogenes. This limited expansion impedes the establishment of clonal PC and the production of large PC batches for clinical applications. In the present study, we used tumor-infiltrating progenitor cells with a high intrinsic expansion capacity to establish stable

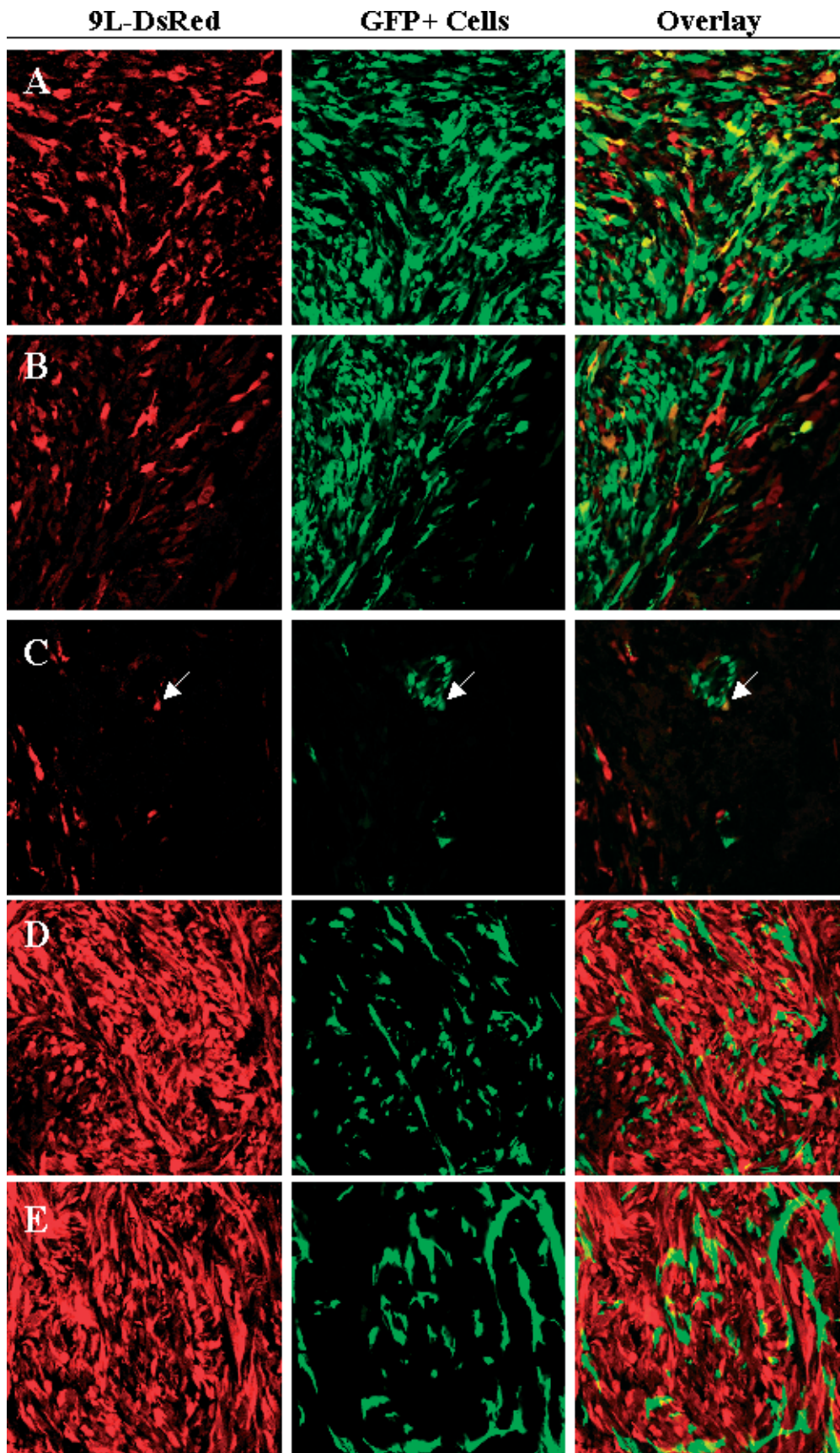


Figure 6. BM-TIPC mediate *in vivo* transduction of rat glioma cells. BM-TIPC were injected intratumorally into established 9L-DsRed gliomas in Fischer rats. After 7 days, rats were killed, brain sections were prepared and analyzed by confocal microscopy. Colocalization of green and red fluorescence demonstrates tumor cell transduction. Shown are images from solid (A) and infiltrating (B, C) tumor areas in which tumor transduction could be readily detected. The white arrow in (C) indicates an infiltrating transduced glioma cell in close proximity to BM-TIPC. Injection of BM-TIC expressing only the gammaretroviral eGFP vector (D) or the gammaretroviral eGFP vector and LCMV-GP (E) did not result in colocalization of green and red fluorescence. Magnification 40 $\times$



PC. Progenitor cells were isolated from adult rat bone marrow according to a protocol established by Jiang and colleagues [15] to isolate multipotent adult progenitor cells (MAPC). MAPC have the capability to differentiate into various cell types *in vitro* and *in vivo*. Moreover, MAPC have a very high expansion capacity and can be expanded to more than 100 population doublings without requiring exogenous immortalization [15]. Although the differentiation capacity of the BM-TIC was not analyzed in this study, marker expression and expansion capacity of BM-TIC strongly resemble that of MAPC. To establish BM-TIC-based PC, BM-TIC were genetically modified to express Mo-MLV Gag-Pol and LCMV-GPopt. The glycoprotein of LCMV was chosen because LCMV-GP mediates up to tenfold higher transduction rates in human glioma cell lines than the amphotropic retroviral envelope protein [16,25]. The expression of viral membrane proteins is often associated with cytotoxicity. Thus, the generation of PC for vectors pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV-G) is hampered by the toxicity of VSV-G and requires inducible expression systems [26]. In contrast to VSV-G, LCMV-GP can be stably expressed in cell lines without causing cytotoxicity [16]. Moreover, LCMV-GP was shown to specifically mediate gene transfer to glioma cells but not to neuronal cells, while VSV-G was found to preferentially target neuronal cells [17]. A lentiviral self-inactivating (SIN) vector encoding LCMV-GP was created and used to transduce bulk cultures of BM-TIC. This led to stable and prolonged LCMV-GP expression in BM-TIC over more than 2 months in culture. Titers achieved on human glioma cells reached  $1 \times 10^4$  TU/ml and are therefore comparable to vector titers of PC used in previous clinical studies [27]. Importantly, as a consequence of tumor infiltration by BM-TIPC, local vector titers throughout the tumor are expected to be higher than titers achieved by injecting non-migratory PC.

When tested for glioma-specific migration *in vivo*, BM-TIPC behaved similarly to the parental BM-TIC cell line (Miletic, Fischer *et al.*, submitted): after a single intratumoral injection, BM-TIPC were detected in most tumor areas, but never in normal brain parenchyma. This shows that BM-TIPC retain their glioma-specific migratory capacity even after extensive culturing, genetic modification and subcloning. One important option for clinical application of BM-TIPC is their injection into gliomas during stereotactic biopsy. The very efficient tumor infiltration by BM-TIPC may then lead to widespread distribution of viral vectors within human gliomas and ensure the targeting of tumor cells distant from the injection site. This targeting of distant tumor cells can currently not be achieved by injection of replication-incompetent vectors due to only limited vector diffusion [6].

In a rat glioma model, transduction was detected in both solid and infiltrating tumor regions, showing that BM-TIPC application is a suitable method to achieve gene transfer into glioma *in vivo*. Interestingly, BM-TIPC colocalized with single invasive glioma cells and mediated

transduction of these cells. Invasive glioma cells migrating into normal brain parenchyma are a major problem in glioma therapy, as they are not accessible to surgery and typically give rise to tumor spread and regrowth. Transduction efficiencies achieved by BM-TIPC injection varied, ranging from undetectable to up to 33% of transduced glioma cells in different areas of the same glioma. In future experiments, the transduction efficiency *in vivo* may be enhanced by using lentiviral vectors instead of Mo-MLV-based gammaretroviral vectors. In contrast to gammaretroviral vectors, lentiviral vectors do not depend on cell proliferation for efficient transduction. Lentiviral gene transfer may thus enhance transduction of tumor areas with low proliferation rate. Specific gene transfer into glioma cells, but not into healthy brain, may thereby be achieved by pseudotyping lentiviral vectors with LCMV-GP [17].

In conclusion, the present study is the first to show that primary adult progenitor cells can be modified to stably produce retroviral LCMV pseudotype vectors while maintaining their high expansion capacity and their specific tropism for gliomas. In the presented model, specificity of gene transfer is conferred by the glioma-specific infiltration of the progenitor cells and by the selective tropism of LCMV-GP-pseudotyped vectors for glioma cells. In future studies, the efficiency of BM-TIPC-mediated gene transfer, shown here for the eGFP marker gene only, needs to be determined for therapeutic genes and extended to human glioma models. Provided that BM-TIPC show a good efficiency in these follow-up experiments, we are confident that these novel progenitor-cell-based packaging cells will be a valuable tool for gene therapy of human gliomas.

## Acknowledgements

The authors would like to thank S. Bracharz, R. Seyd, M. Carstov and T. Merovci for expert technical assistance and F. Hermann for helpful discussions. The Neural Regeneration Group at the University Bonn LIFE & BRAIN Center is supported by the Hertie-Foundation and Walter-und-Ilse-Rose-Foundation. This work was additionally supported by the Köln Fortune Program (grant 108/2003 to H.M.). This publication was generated in the context of the *CellPROM* project, funded by the European Community as Contract No. NMP4-CT-2004-500039 under the 6th Framework Programme for Research and Technological Development in the thematic area of 'Nanotechnologies and nano-sciences, knowledge-based multifunctional materials and new production processes and devices.' This publication reflects only the authors' views; *CellPROM* is not liable for any use that may be made of the information contained therein.

## Supplementary material

The supplementary electronic material for this paper is available in Wiley InterScience at: <http://www.interscience.wiley.com/jpages/1099-498X/suppmat/>.

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